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EFFECTS OF ORGANOTINS ON GROWTH AND SURVIVAL OF TWO MARINE DIATOMS, SKELETONEMA COSTATUM AND THALASSIOSIRA PSEUDONANA

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ABSTRACT

Tributyl- and triphenyltin compounds inhibited population growth and cell survival of marine unicellular algae at low concentrations. They may pose a threat to algae in areas of industrial outfalls and heavy boat traffic.

INTRODUCTION

Organotin compounds are used extensively as flame resistant polymers, wood preservatives, pesticides, slimicides, water resistant coatings, disinfectants, antioxidant inhibitors, corrosion inhibitors in lubrication oils, catalysts for polymerization of olefins, thermal stabilizers in polyvinyl chloride (PVC) products, preservatives in adhesives, in latex paints, in the manufacture of solder, and as cataylsts in reduction of polyurethane foams. Tributyltin oxide (TBTO) and tributyltin fluoride (TBTF) are used as biocides in marine antifouling paints. Total production of 10 industrial alkyltin compounds in the United States increased from approximately 2.3 million kg in 1965 to 10.4 million kg in 1976; production by 1986 was estimated to be approximately 25 million kg[1]. Thirty-three alkyltin compounds were listed by the U.S. Environmental Protection Agency (EPA)[2] as being commercially important. Most were methyl-, ethyl- and butyltins.

Although EPA[3] stated that release of organotins from manufacturing and fabrication plants, leaching from PVC, release after polymer catalyst use or after application as pesticides did not constitute substantial sources, these compounds have been detected in aquatic ecosystems. Organotin compounds have been found in effluents of manufacturing plants

and after use as disinfectants[4]. Concentrations of TBTO up to 0.06 µg l^{-1} have been reported from San Diego Bay[5], and water samples from two nearby boat basins averaged 0.1 and 0.16 µg l^{-1} [5]. Such concentrations were toxic to the copepod, Acartia tonsa[5]. In France, concentrations of TBTO and TBTF were high enough to be toxic to oyster larvae[6]. In both the U.S. and France, leaching from marine antifouling paints was attributed to be the main source of organotin pollution.

Little is known about the natural origin of organotins in aquatic ecosystems. Biomethylation is a major source of methyltins[7,8,9], although there is an apparent relationship between runoff, anthropogenic activity and concentration gradients of methylstannanes in Chesapeake Bay and Baltimore Harbor (USA)[7]. Methylated species in water may also be derived from atmospheric fallout[7]. Mono-, di- and tributyltins were found in subsurface waters in Canada[8], but organic and inorganic tin species were concentrated in the surface microlayer, with highest concentrations near chemical plants and in harbors with heavy shipping traffic[8].

Environmental degradation of organotins has been reviewed [1,10,11]. Generally, these compounds degrade slowly in water through photochemical and microbiological decomposition. Time for degradation of 50% of TBTO in water exceeded 89 days[12]. Organotin compounds may also be inactivated when adsorbed to particles suspended in water. Half of the TBTO on unsterile soil was lost in 15 to 20 weeks, whereas much less was lost from sterile soil[13]. Conversion was to dibutyltin derivatives and carbon dioxide, and a much longer time was postulated for complete mineralization of the parent compound. Forty-one weeks were required for adaptation of organisms in sewage sludge before significant degradation of TBTO was detected[4].

Toxic properties or organotins were recognized over 130 years ago, and their effects on aquatic organisms have been summarized[11]. Generally, trialkyl compounds are more toxic than others, and those with butyl and phenyl groups are more toxic than those with methyl, ethyl, propyl and octyl groups. For animals exposed to TBTO, 0.15 µg l^{-1} caused thickening of the shells of oysters[15], 0.5 µg l^{-1} caused increased weight of the megalopa of Rithropanopeus harrisii (crab)[16], 1 µg l^{-1} was acutely toxic to larvae of Homarus americanus (lobster)[17] and 2 µg l^{-1} was the 96-h LC50 with Nitocra spinipes (copepod)[18]. In studies with the freshwater alga, Ankistrodesmus falcatus, IC50s were: dimethyltin, 0.57 mg l^{-1}; diethyltin, 0.10 mg l^{-1}, tripropyltin, 0.014 mg l^{-1}; tributyltin, 0.005 mg l^{-1}, and triphenyltin, 0.002 mg l^{-1} [14]. A direct relationship between toxicity and the octanol-water partition coefficient was also demonstrated[14].

This paper reports effects of stannous chloride and 21 organotin compounds on growth and survival of two species of marine unicellular algae, Skeletonema costatum (Greville) Cleve and Thalassiosira pseudonana Hasle and Hundal. Possible effects upon algal populations in contaminated waters are discussed.

MATERIALS AND METHODS

The two centric diatoms, S. costatum and T. pseudonana, were obtained from the culture collection of the University of Rhode Island, Kingston. Stock cultures were maintained in

artificial seawater medium fortified with trace metals, vitamins and nutrient salts[19].

Two methods were used for estimation of effects of tin compounds on algae. In the first, a test of algal population growth, 50 ml of artificial seawater growth medium were added to Erlenmeyer culture flasks of 150 ml capacity that were capped with stainless steel closures and sterilized by pasteurization. Salinity of the medium was 30 g l^{-1} and pH was 8.1 after sterilization. Algal stock cultures in the logarithmic phase of growth were diluted to yield an initial cell concentration of approximately 2.5 X 10^3 cells ml^{-1} when one ml was added to 50 ml of test medium. Toxicant was added in 0.01 ml nanograde acetone (final concentration of acetone = 0.02%) from an Eppendorf pipette. Five concentrations of toxicant were used in each test. Each concentration, untreated control and acetone control was replicated three times within each test, and each test performed one to three times. Algae were incubated for 72 h on shakers (60 excursions min^{-1}) under 150 $\mu E\ m^{3\ -1}\ sec^{-1}$ of photosynthetically active radiation from cool white fluorescent tubes in a culture room. Temperature was 20 ± 0.5 C and the lighting cycle was 14 h light: 10 h darkness. Algae were enumerated on a hemocytometer and the concentration that would inhibit growth by 50% (EC50) was estimated by the moving average angle method[20]. In algal toxicity tests, concentration response relationships differ from animal tests, for which the moving average angle test was derived, because the number of individuals at the end of the test may be greater than at the beginning. The authors are aware of the assumptions for the moving average angle method, and some liberty has been taken in applying it to algal data. However, the calculated concentrations that would inhibit growth by 50% derived here are intended only to express relative toxicity, and confidence intervals calculated by the moving average angle method are not given. It was assumed that the average cell count of the controls (N_o) would be expected in treated algal populations if no effect occurred within the same test. The estimated population size, $\ddot{p}$, was determined to be

$$\ddot{p} = \begin{cases} 1 - \frac{s}{N_o} & \text{if } s \leq N_o \\ 0 & \text{if } s > N_o \end{cases}$$

where s is the average number of cells counted ml^{-1} within an exposure group. Estimates of the EC50s were computed by using the three point moving average angle method[20].

The second test was designed to estimate concentrations of organotins that were lethal to S. costatum. Algae from inoculum stock cultures were added to 50 ml of growth medium as described above to yield a final concentration of approximately 2.5 X 10^3 cells ml^{-1}. Growth of cultures proceeded under the conditions described above for 48 h, after which, a 5 ml aliquot was removed asceptically from each flask and its fluorescence measured on a Perkin-Elmer Model 210 fluorescence spectrophotometer[1] (exciter wavelength, 430 nm; analyzer wavelength, 672 nm). Five concentrations of each test compound in 0.01 ml nanograde acetone were added to duplicate flasks, along with duplicate untreated control and acetone control flasks.

[1]Reference to trade names does not imply endorsement by the U.S. Environmental Protection Agency

The cultures were returned to the culture room and 5-ml samples removed asceptically for fluorescence analysis at 24, 48 and 72 h after addition of toxicant. After 72 h of exposure, 5 ml were pipetted into test tubes and dead cells stained with Evans blue[21]. Five hundred cells were counted and classified as living or dead for each culture. Therefore, a total of 1,000 cells were examined for each concentration. Results are expressed as the percentage of dead cells in each concentration. The EC50 and LC50 (calculated concentration lethal to 50% of the population) were calculated by extrapolation[22].

An experiment was performed to learn if S. costatum and T. pseudonana could adapt to TPTO and TBTO. The algae were exposed initially to 0.2 µg l^{-1} of each compound, allowed to grow for one week under the conditions described above, and transferred serially to this and higher concentrations each week for 12 weeks. After 12 weeks, highest concentrations that did not suppress growth by 100% were: 4 µg l^{-1} TPTO (T. pseudonana), 1 µg l^{-1} TPTO (S. costatum), 4 µg l^{-1} TBTO (T. pseudonana) and 6 µg l^{-1} TBTO (S. costatum). These concentrations provided enough cells for growth tests as described above, and population size was estimated by fluorescence spectroscopy. Untreated cell cultures were tranferred serially in a way identical to exposed cultures and served as controls.

Concentrations of tin compounds in stock solutions were estimated by measurement of elemental tin. Only stock solutions were analyzed for accuracy of tin concentrations because those in toxicity tests were generally below the limit of detection by inductively coupled argon plasma (ICAP) techniques.

Stock solutions of organotin compounds in acetone (5 ml) were evaporated to dryness with a gentle stream of nitrogen and resolubilized in 2 ml of 1:1 HNO_3 and water. For volatile tin compounds such as tetraethyltin, acid was added before the acetone was evaporated. Samples were digested on a steam bath for at least 8 h and diluted with distilled water before analysis.

The samples were analyzed on an ICAP spectrophotometer, Jarrell-Ash Model 800 Atom Comp[R] equipped with spectrum shifter background correction, Digital 8a computer, Tylan[R] mass flow controller and fixed cross flow nebulizer. The ICAP was operated at 1.0 Kw forward power, with 20 l min^{-1} argon coolant and 0.6 l min^{-1} sample flow. The observation height was 16 mm above the load coil. Tin was quantified at the 2839 Å line of the polychromater after the instrument was calibrated with a 10 mg l^{-1} solution of Baker Instra Analyzed atomic spectral standard. The average of four consecutive burns was calculated. The original tin stock concentration was calculated by dividing the tin concentration by the percentage tin in the molecule. Blanks and standards were analyzed frequently throughout the analytical procedure to detect possible contamination or drift.

Detection limits for tin, calculated as three times the standard deviation for 10 burns of a blank, ranged from 50 to 100 µg l^{-1} over the period of study. Instrumental response to tin was linear from the detection limit to 1,000 mg l^{-1}.

Only tests in which the concentrations of organotins in stock solutions were accurate (± 3%) are reported.

[R]Fisher Scientific Co., Jarrell-Ash Division, Waltham, MA; Tylan Corp., Carson, CA

RESULTS AND DISCUSSION

Growth studies. The average EC50 for each compound is given in Table 1. Inorganic tin, as stannous chloride, was only slightly toxic: the EC50s were 325 $\mu g\ l^{-1}$ for S. costatum and 316 $\mu g\ l^{-1}$ for T. pseudonana. In the methylated tin group, methyltin trichloride was toxic to S. costatum. Its EC50 of 78 $\mu g\ l^{-1}$ was lower than those of most herbicides tested with marine unicellular algae, e.g. atrazine (100-300 $\mu g\ l^{-1}$) and monuron (100-150 $\mu g\ l^{-1}$)[23]. Since methylation of tin is known to occur in aquatic ecosystems[7,9], toxic concentrations of methyltin may be formed in areas of anthropogenic input of organic and inorganic tin.

Although diethyltin was not inhibitory to algal growth at the concentrations tested,

Table 1. Average EC50 values of tin compounds and Skeletonema costatum and Thalassiosira pseudonana after exposure for 72 h.

Compound	S. costatum		T. pseudonana	
	No. of Tests	$\bar{x}$ EC50 $\mu g\ l^{-1}$	No. of Tests	$\bar{x}$ EC50 $\mu g\ l^{-1}$
Stannous chloride	1	325	2	316
Methyltin trichloride	3	78	2	342
Dimethyltin dichloride	1	>500	1	>500
Trimethyltin chloride	2	214	2	348
Tetramethyltin	1	>500	1	>500
Diethyltin dichloride	1	>500	1	>500
Triethyltin bromide	-	-	2	3.8
Tetraethyltin	2	142	2	127
Di-n-butyltin difluoride	3	56	2	283
Dibutyltin dichloride	1	40	1	181
Dibutyltin diacetate	3	35	1	127
Tributyltin acetate	2	0.36	2	1.28
Bis(tri-n-butyltin) oxide	2	0.33	1	1.03
Tri-n-butyltin chloride	2	0.36	-	-
Tributyltin fluoride	1	>0.25<0.50	-	-
Tetrabutyltin	2	17	-	-
Phenyltin trichloride	1	>500	1	>500
Diphenyltin dichloride	2	31	2	34
Triphenyltin acetate	3	0.86	2	1.09
Bis(tripnenyltin) oxide	3	0.81	3	1.25
Triphenyltin chloride	2	0.92	2	1.34
Triphenyltin hydroxide	2	0.59	1	1.07

triethyl- and tetraethyltin were considerably more toxic than methylated tin. The EC50 of triethyltin was 3.8 μg l^{-1} when tested with T. pseudonana, approximately 0.01 that of the methyltins.

As a group, the butyltins were most toxic. Within the group, tributyltins were the most toxic, with similar EC50s of approximately 0.35 μg l^{-1} for S. costatum and 1.15 μg l^{-1} for T. pseudonana.

Di- and triphenyltins were also highly toxic to the algae. The triphenyls were most toxic: average EC50s were approximately 0.8 μg l^{-1} for S. costatum and 1.19 μg l^{-1} for T. pseudonana.

In all cases, except stannous chloride, where toxicity was approximately equal, S. costatum was more sensitive than T. pseudonana.

These results with organotins with various alkyl groups are similar to those reported for 4 freshwater algal species[14], except that the marine species are more sensitive. Also, methyltin, which was not highly toxic to freshwater algae, was very toxic to S. costatum.

Cell death. The tin compounds that were most toxic in growth tests also killed S. costatum cells exposed to low concentrations (Table 2). The EC50s reported for tests on cell death are greater than those of growth tests because biomass at the time of exposure was greater.

The type of response given to the most toxic compounds is illustrated in Fig. 1 by TBTO. Living biomass, as estimated by height of the fluorescence peak, decreased in concentrations between 7.5 and 50 μg l^{-1} 24 h after exposure, with recovery by 48 or 72 h (Fig. 1A). Depression of the fluorescence response was caused, at least in part, by death of cells. There was a steep rise in percentage of dead cells in cultures exposed to between 10 and 25 μg l^{-1} and a gradual increase to near 100% at 50 μg l^{-1} TBTO after 72 h of exposure.

EC50 and LC50 values were close. The LC50s of the 9 compounds for which they could be derived averaged 2.8X (range 1.2 - 8.2X) greater than the EC50s. By extrapolation from Fig. 1B, 50.5% of the cells were dead at the EC50 concentration. Extensive death of cells at or near the EC50 concentration may indicate greater hazard than when the LC50 far exceeds the EC50.

These data show that populations of algae exposed to relatively high concentrations of organotins (up to 50 μg l^{-1}) over a short period of time may recover without input of new cells. However, the data do not show whether recovery was due to adaptation of surviving cells or loss of toxicant from the medium by volatilization, adsorption to dead cells or to walls of exposure vessels, degradation or a combination of these factors.

Adaptation experiments. Algae did not adapt to the presence of TPTO or TBTO after exposure through 12 serial transfers for 12 weeks. Table 3 shows that cells which survived in concentrations above the EC50s of growth tests did not have higher EC50s than cells that were never exposed. Development of tolerence by algae to ionic forms of heavy metals has been described and related to rapid adaptation in the laboratory[25]. Lack of adaptability in our tests was surprising, since it had been shown that the freshwater alga, A. falcatus, possesses

Table 2. EC50 and LC50 values for selected tin compounds tested with Skeletonema costatum. Exposure of algae was made after 48 h growth, and EC50 and LC50 values calculated after 72 h exposure.

	$\mu g\ l^{-1}$	
Compound	LC50	EC50
Stannous chloride	>500	>500
Methyltin trichloride	>500	>500
Trimethyltin chloride	>500	>500
Triethyltin bromide	40.2	22.5
Tetraethyltin	>500	>500
Dibutyltin dichloride	>500	265
Dibutyltin diacetate	>500	304
Tributyltin acetate	14.7	9.3
Bis(tri-n-butyltin) oxide	14.2	11.4
Tri-n-butyltin chloride	11.5	5.6
Tributyltin fluoride	11.9	9.3
Tetrabutyltin	>500	186
Diphenyltin dichloride	>500	228
Triphenyltin acetate	16.8	5.4
Bis(triphenyltin) oxide	4.3	2.4
Triphenyltin chloride	13.8	3.6
Triphenyltin hydroxide	13.9	1.7

a mechanism for sequential debutylation of TBTO[26]. Presumably, the most likely method of adaptation of algae would be by elaboration of a debutylation mechanism in exposed populations. It has been suggested that development of resistance may be an important and widespread response of marine organisms to pollutants in nature, but that not all planktonic species may have the required genetic mechanisms[27]. Our data indicate that adaptation of at least some algal species to organotins in the field is unlikely.

In summary, tributyl- and triphenyltins are highly toxic to algae in 72 h, and EC50s were lower than many LC50s derived from animal tests reported in the literature. The only substance that we know to be more toxic in algal growth tests is the insecticide tetrahydro-5-5-dimethyl-2(1H)-pyrimidinoine[3-[4-(trifluoromethyl)phenyl]-1-[2-[-4(trifluoromethyl) phenyl]ethenyl]-2-propenylidene hydrazone[24]. However, this insecticide is rapidly degraded by light, whereas alkyltins may be expected to have relatively long residence times in aquatic ecoysystems. Although recovery occurred in vitro when cell density was high (ca. 4.25×10^3

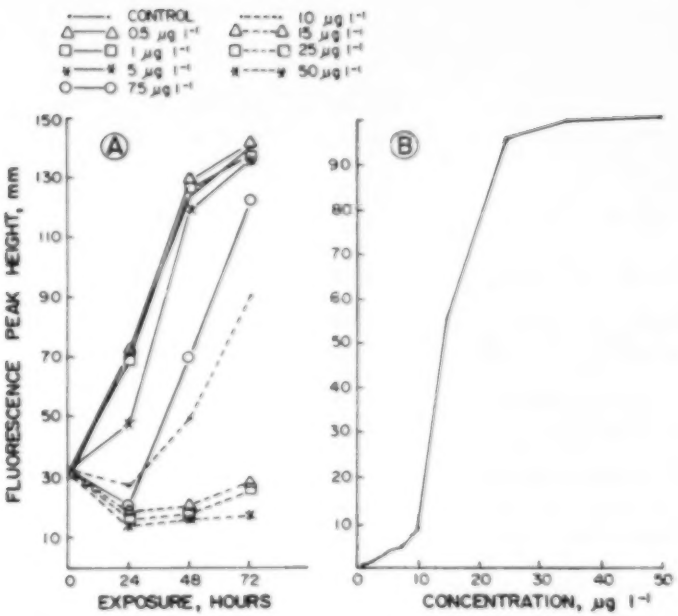

Figure 1. Effects of TBTO on growth and survival of Skeletonema costatum. A - Population growth; B - Death of cells

Table 3. Responses of Skeletonema costatum and Thalassiosira pseudonana after 12 serial transfers to increasing concentrations of TBTO and TPTO.

Species	Final Exposure conc.	72 h EC50 µg l^{-1}
S. costatum	Control	0.52
	6 µg l^{-1} TBTO	0.65
T. pseudonana	Control	0.97
	4 µg l^{-1} TBTO	0.84
S. costatum	Control	0.59
	1 µg l^{-1} TPTO	0.68
T. pseudonana	Control	0.84
	4 µg l^{-1} TPTO	0.62

cells $ml^{3\ -1}$ after 48 h growth), a relatively large number of cells was killed at the EC50 concentrations: maximum cell deaths with 4 pesticides in previous tests never exceeded 3.3% after exposure for 72 h^{25}. In addition, adaptation to TPTO and TBTO could not be demonstrated in the laboratory. These findings indicate that organotins may pose a threat to survival of algae in contaminated ecosystems.

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